AD________________

Award Number: DAMD17-01-1-0525

TITLE: Detection of Mitochondrial DNA Mutations in Mammary Epithelial Cells in Nipple Aspirate Fluid

PRINCIPAL INVESTIGATOR: Bassem R. Haddad, M.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (1 Sep 2002 - 31 Aug 2003)

4. TITLE AND SUBTITLE
Detection of Mitochondrial DNA Mutations in Mammary Epithelial Cells in Nipple Aspirate Fluid

5. FUNDING NUMBERS
DAMD17-01-1-0525

6. AUTHOR(S)
Bassem R. Haddad, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

E-Mail: haddadb1@georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Because optimal methods have not been established for screening and early detection of breast cancer in premenopausal women, there is an urgent need for a variety of new approaches that can augment mammographic screening.
We describe a method for the idolation of free DNA from ductal lavage (DL) and nipple aspirate fluid (NAF), and its evaluation for the presence of mitochondrial DNA (mtDNA) mutations at the D310 marker, to improve early detection of breast cancer. We evaluated 26 DL and six NAF samples form 14 women of known BRCA1 status, who have no clinical evidence of breast tumors: nine mutation carriers and five non-carriers. The mitochondrial studies were possible in all 26 DL samples and a somatic mutation was found in 3/9 carriers and in none of the non-carriers. mtDNA mutation evaluation was possible in 4/6 NAF samples. The NAF and DL results were concordant. One NAF sample from a BRCA1 patient showed a mtDNA mutation. Our data demonstrates the feasibility of performing these studies using the free DNA present in the ductal fluid, while the intact cells can be used for cytologic studies.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

It is widely believed that the accumulation of genomic aberrations occurs very early in the process of mammary tumorigenesis and may precede morphologic changes [1, 2, 3]. Therefore, development of methods which permit the reliable identification of these early genetic changes in mammary cells obtained through non-invasive or minimally invasive approaches such as ductal lavage or nipple aspiration, will greatly benefit early detection of breast cancer. Our group has recently made an important observation while working on another project related to the characterization of early genomic changes in mammary glands of high risk women, a project funded also by the DOD breast cancer program (DAMD17-99-1-9193). We have found that genomic changes in the form of LOH at relevant markers occurs not only in histologically abnormal and malignant breast tissues, but also in morphologically normal tissues as well as in areas with pathologically benign changes. This is the first study to demonstrate genetic changes in the form of LOH in morphologically normal mammary tissues and in benign tissues from breast cancer patients that are carriers of mutations in the *BRCA1* and *BRCA2* genes. Such changes may represent the earliest detectable genomic aberrations that occur during the development and progression of breast cancer in these high-risk patients. These findings are currently in press, in *Cancer Genetics and Cytogenetics.* [1]. (A copy of the manuscript is included with this report, for your review). The implications of these findings for early detection of breast cancer in high-risk women are clear. Specifically, the ability to detect genetic alterations in the ductal fluid obtained through nipple aspiration or lavage, will offer an excellent opportunity to improve early detection of the disease. But why look in the ductal fluid?

The optimal method for early detection of breast cancer in premenopausal women is not known. Currently, mammographic screening is the best available approach for early detection in the general population. However, mammography alone may not be sufficient in young women, for the following reasons: (a) effectiveness of mammography has not been established in women younger than 40 since younger women have more dense breast tissue, which reduces mammographic sensitivity [4], (b) tumor growth rates may be higher in younger women thereby necessitating frequent screening, and (c) carriers of some mutations (such as ataxia telangiectasia) may have increased sensitivity to radiation and conceivably could be harmed by frequent mammograms [5]. Therefore, there is a critical need to develop screening methods to augment mammography for the early detection of breast tumors, particularly in high-risk women, such as *BRCA1* or *BRCA2* mutation carriers, who face markedly elevated risks of developing early onset breast cancer. One approach would be the use of molecular markers associated with neoplastic changes in a screening strategy for early detection. But what molecular markers to use?

Recent studies have shown the presence of several mitochondrial DNA (mtDNA) mutations in a variety of tumor types, including breast, colorectal, bladder, head and neck, and lung tumors [6, 7, 8]. A mtDNA mutation at the microsatellite marker D310, a mononucleotide repeat of 300–315 nucleotides which is located at the D-loop region of mitochondrial DNA and involved in the mtDNA replication process, was reported to be altered in about 30% of breast cancers. Mutations at this region are common in primary

human tumors, including breast cancer and are likely to have functional relevance in tumor development [9]. In addition, and as described above, LOH is both an early and a common event in breast cancer progression [1]. Therefore, development of a method to detect genomic aberrations in mammary epithelial cells is an exciting opportunity for improving the detection of breast cancer at an early stage, particularly if performed on cells obtained through non-invasive or minimally-invasive approaches such as nipple aspiration (NAF) and ductal lavage (DL).

Nipple aspirate fluid (NAF) has been studied for many years as a non-invasive method to examine changes in breast biology and to identify women with high risk of breast cancer or preclinical disease [10]. NAF is secreted continuously by the non-lactating breast and, in 50-70% of premenopausal women, it can be aspirated through ductal openings in the nipple using a simple, non-invasive pump. NAF is of interest because it has a relatively long retention time in the breast alveolar-ductal system where it accumulates exfoliated mammary epithelial cells [10, 11, 12]. Evaluating these cells may provide not only a "snapshot" of the micro-environment where breast cancer originates, but also a realistic opportunity to improve the detection of breast tumors at their earliest stages. However, a major hurdle impeding the success of our assays is the low cellularity and low volume of NAF samples. The typical NAF sample from a woman with no breast abnormalities is about 10 μl in volume and contains fewer than 10 ductal epithelial cells [10]. One very promising new approach to overcoming these two issues has recently been designed and tested: Breast Ductal Lavage.

Ductal lavage [13] is a new, minimally invasive technique developed to evaluate the ductal fluid and cells. A small catheter is inserted into the duct. Saline is instilled and the breast is massaged to dislodge cellular material lining the duct. The fluid is then collected for analysis. In a recent study [13], ductal lavage was performed on greater than 500 high-risk women at 19 breast cancer centers. Atypical cells were seen in 17% of patients and suspicious or malignant cells were identified in 7% of patients. The lavage procedure also resulted in specimens sufficient for cellular analysis in a far greater number of patients than did nipple aspiration; in fact, ductal lavage produced an average of 40,000 cells per duct. This high yield of cells makes it much more likely that sufficient cells would exist for both cytologic analysis and for genetic studies. In addition, this procedure allows the physician to pinpoint the specific duct from which the abnormal cells originated, so that a specific area of the breast can be more thoroughly evaluated and more closely followed.

Body:

In this project, we are investigating the possibility of using the ductal lavage fluid and the nipple aspirate fluid to detect mitochondrial DNA mutations as an early sign of breast cancer and to determine the optimal method for detection of theses mutations. We have initiated an active collaboration with Dr. David Sidransky and his group at Johns Hopkins University to study mtDNA mutations in the ductal fluid. Dr. Sidransky's group discovered that mtDNA mutations at the microsatellite marker D310 in the DL and NAF fluids is present in over 30% of breast cancers [9]. Our working hypothesis is that women with early-stage breast cancer may have mtDNA mutations in mammary epithelial cells

shed into the nipple aspirate fluid (NAF) and the ductal lavage (DL) fluid which can be used as a marker for screening and detection of early disease. In addition, we hypothesize that mtDNA mutations in NAF and DL-derived cells are representative of those found in tumor tissue in the same breast. Through our collaboration with Dr. Sidransky, we were successful to set up the assay for the detection of mutations at the D310 marker in the DL and the NAF fluids.

Use of the free DNA isolated from NAF and DL:

We developed an approach that allows the isolation of free DNA from the lavage fluid, thereby leaving the entire cellular content for cytological analysis. This approach offers the distinct advantage of allowing the cytopathologist to evaluate all the recovered cells, while enough free DNA can be isolated and used for PCR based assays. We have also successfully isolated free DNA from the NAF fluid for molecular evaluation by PCR. The isolated DNA was used to detect mitochondrial DNA (mtDNA) mutations.

mtDNA mutation analysis in DL and NAF samples:

We have performed ductal lavage on a total of 14 subjects: nine *BRCA1* carriers and five true-negatives. Of these, seven had adequate material for cytologic analysis and only one, a *BRCA1* mutation carrier, demonstrated focal minimal atypia. The remainder had benign findings. A total of 26 DL specimens were obtained.
The DL specimens were evaluated for mitochondrial DNA mutations at the microsatellite marker D310, located at the D-Loop region using a PCR based assay. The mitochondrial studies were possible in all 26 DL samples. In three of the carriers, D310 length difference between the DL samples and the corresponding blood sample (somatic mutation) was found. No mtDNA mutations were detected in the true-negative subjects. Figure 1a shows examples of DL samples with no mtDNA mutations and Figure 1b shows an example of a DL sample with a mtDNA mutation. We also evaluated six NAF samples from four patients (two carriers and two non-carriers) for mitochondrial mutations. The evaluation was possible in four of the six samples. A mutation was detected in one sample from a carrier whose DL from the same duct also showed the same mutation (Figure 1b). Three samples had no evidence of mutations, and no mutations was seen in their corresponding DL samples either. In all four NAF samples, the mitochondrial DNA findings were consistent with the corresponding DL findings. The patients with mitochondrial abnormalities in the DL fluid had normal physical exam, a normal mammogram and normal findings on FDG-PET scan. The patients continue to be closely monitored.

LOH analysis of the DL and NAF fluids:

Because of the very exciting results that we obtained from our other DOD funded project [1], namely the detection of genetic changes in the form of LOH in morphologically normal mammary tissues and in benign tissues from breast cancer patients that are carriers of mutations in the *BRCA1* and *BRCA2* genes, described above, we plan to

perform LOH at relevant markers, on the same fluid specimen used for the mitochodrial studies. The ability to conduct more then one molecular assay on the same fluid sample offers a tremendous advantage for early detection of breast cancer, as it increases the chances to detect an early molecular change which in turn may indicate the presence of an early cellular transformation.

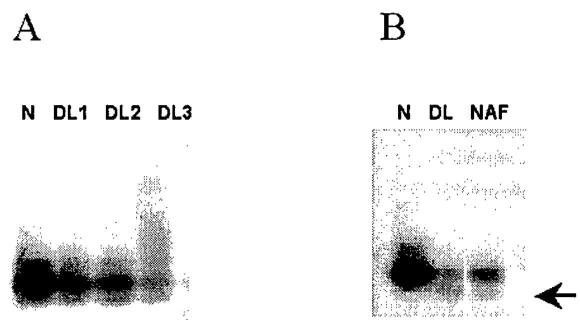


Figure 1: Analysis of the D310 marker in DL samples from two different patients. Figure 1a shows the analysis of the D310 marker in three DL samples obtained from three different ducts from the same patient, a non-carrier of the *BRCA1* mutation (Lanes DL1, DL2, DL3). No mtDNA mutation was found. Lane N shows the D310 pattern in the blood. Figure 1b, shows a mutation (arrow) at the D310 marker observed in a DL sample (Lane DL) and in the NAF sample (Lane NAF) from another case, a *BRCA1* carrier. Lane N shows the D310 pattern in the blood of that patient.

Key Research Achievements:

-Optimized conditions to detect mtDNA mutations in the D310 marker in DL and NAF.
-Obtain NAF and DL specimens from 14 patients: nine *BRCA1* mutation carriers and five negative controls.
-Successfully evaluate 26/26 DL samples and 4/6 NAF samples.

Reportable outcomes: N/A

Conclusions:

We plan to perform mitochondrial studies on additional fluid specimens. Furthermore, because of the very exciting results mentioned above, we would like to perform other molecular studies, namely LOH at relevant markers, on the same fluid specimen used for the mitochodrial studies. The ability to conduct more then one molecular assay on the same fluid sample offers a tremendous advantage for early detection of breast cancer, as it increases the chances to detect an early molecular change which in turn may indicate the presence of an early cellular transformation. We believe that testing this approach is critical to establish its proof of principle before a larger study can be proposed.

References:

1- Cavalli LR, Singh B, Isaacs C, Dickson RB, Haddad BR: Loss of Heterozygosity in normal breast epithelial tissue and benign breast lesions in *BRCA1/2* carriers with breast cancer. Cancer Genet Cytogenet *in press.*
2- O'Connell P, Pekkel V, Fuqua S, Osborne CK, Allred DC: Molecular genetic studies of early breast cancer evolution. Breast Cancer Res Treat 32:5-12, 1994
3- Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS: Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science 274:2057-2059, 1996
4- Rimer BK. Breast cancer screening. In: Diseases of the Breast, Harris JR, Lippman ME, Morrow M, Hellman S, eds. Lippincott-Raven Publishers, Philadelphia, pps. 307-322, 1996.
5- Swift M. Ionizing radiation, breast cancer, and ataxia-telangiectasia. J Natl Cancer Inst 86:1571-1572, 1994.
6- Polayk K, Li Y, Zhu H, Lengauer C, et. al. Somatic mutations of the mitochondrial genome in human colorectal cancer. Nature Genet 20:291-293, 1998.
7- Fliss MS, Usadel H, Caballero OL, Wu L, et. al. Facile Detection of Mitochondrial DNA mutations in tumors and bodily fluids. Science 287:2017-2019, 2000.
8- Parrella P, Xiao Y, Fliss M, et al. Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. Cancer Res. 61:7623-7626, 2001.
9- Sanchez-Cespedes M, Parrella P, Nomoto S et al. Identification of a mononucleotide repeat as a major target for mitochondrial DNA alterations in human tumors.Cancer Res. 61:7015-7019, 2001.
10-Petrakis NL. Nipple aspirate fluid in epidemiologic studies of breast disease. Epidemiologic Reviews 15:188-195, 1993.
11-Petrakis NL. Physiologic, biochemical, and cytologic aspects of nipple aspirate fluid. Breast Cancer and Treatment 8:7-19, 1986.
12-Petrakis NL. Studies on the epidemiology and natural history of benign breast disease and breast cancer using nipple aspirate fluid. Cancer Epidemiol Biomark Prev 2:3-10, 1993.
13- Dooley WC, Ljung BM, Veronesi U, Cazzaniga M, et. al. Ductal lavage for detection of cellular atypia in women at high risk for breast cancer. J Natl Cancer Inst 93:1624-1632, 2001.

Appendix:

Cavalli LR, Singh B, Isaacs C, Dickson RB, Haddad BR: Loss of Heterozygosity in normal breast epithelial tissue and benign breast lesions in *BRCA1/2* carriers with breast cancer. Cancer Genet Cytogenet *in press.*

ELSEVIER

Cancer Genetics and Cytogenetics ■ (2004) ■

CANCER GENETICS AND CYTOGENETICS

Loss of heterozygosity in normal breast epithelial tissue and benign breast lesions in *BRCA1/2* carriers with breast cancer

[AQ1]

Luciane R. Cavalli[a,b,c], Baljit Singh[a,b,d], Claudine Isaacs[a,b,e], Robert B. Dickson[a,b], Bassem R. Haddad[a,b,c,f,*]

[a]*Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007*
[b]*Department of Oncology, Georgetown University Medical Center, Washington, DC 20007*
[c]*Institute for Molecular and Human Genetics, Georgetown University Medical Center, 3800 Reservoir Road NW, Main 4000, Washington, DC 20007*
[d]*Department of Pathology, Georgetown University Medical Center, Washington, DC 20007*
[e]*Department of Internal Medicine, Georgetown University Medical Center, Washington, DC 20007*
[f]*Department of Obstetrics and Gynecology, Georgetown University Medical Center, Washington, DC 20007*

Received 22 April 2003; received in revised form 2 July 2003; accepted 10 July 2003

Abstract

Loss of heterozygosity (LOH) of the wild-type *BRCA1/2* allele is a reproducible event in breast tumors of *BRCA1/2* mutation carriers, but it is unknown if this allelic loss occurs only in association with recognizable histopathologic abnormalities. We evaluated the early genomic changes that occur in the mammary glands of patients with increased predisposition to breast cancer due to germline mutations in the *BRCA1/2* genes. We tested the hypothesis that these genomic changes may be detected, not only in histologically abnormal and malignant breast tissues, but also in morphologically normal tissues and in areas with pathologically benign changes. Samples were obtained from five breast cancer patients: four *BRCA1* carriers and one *BRCA2* carrier. In each case, nontumor tissue areas surrounding the tumor or from other locations of the breast were isolated using laser capture microdissection. We evaluated 29 areas showing normal terminal ductal lobular units (TDLUs) or histopathologically benign changes (in particular, sclerosing adenosis), using a panel of polymorphic dinucleotide microsatellite markers for the *BRCA1* gene and other chromosome 17 loci, for the *BRCA2* gene and other chromosome 13 loci, and for the *FHIT* gene on chromosome [AQ2] 3p14.2. Overall, we performed a total of 105 analyses; LOH was detected in 59 of the 105 (56%). In the normal TDLUs, 15 of 30 analyses (50%) showed LOH; in the tissues with benign proliferative changes, such as sclerosing adenosis, 44 of 75 analyses showed LOH (59%). Our results suggest that there is a field effect of early genetic events preceding morphologic changes in the mammary glands of *BRCA* mutation carriers.

1. Introduction

The onset and progression of breast cancer involves progressive deviations from normal control mechanisms of cellular proliferation, followed by selection and highly abnormal regulation of both processes as genomic instability increases. Breast carcinomas are known to display a high degree of intratumor genetic heterogeneity, without any obvious correlation to their morphologic appearance. Breast cancer progression is associated with genomic instability where loss of heterozygosity (LOH) is a key mechanism of inactivation of the wild-type allele, corresponding to recessive, loss-of-function mutations in tumor suppressor genes [1]. Because many patients with hereditary breast cancer carry a mutation in one of two known tumor suppressor genes, *BRCA1* on chromosome 17 or *BRCA2* on chromosome 13, LOH of the wild-type *BRCA* allele may be the first genetic event that occurs in the mammary glands of these patients beginning carcinogenic progression [2–5]. Although this type of pivotal initial genetic event is likely, given the highly reproducible observation of *BRCA* LOH in tumors of carriers of *BRCA* mutations, any correlation of the initial LOH event with specific, early histopathologic changes remains completely unknown. If *BRCA* genes are haploid sufficient and act as gatekeepers of genetic alterations in mammary epithelial cells, then LOH of *BRCA* loci would be expected to correlate with onset of an early epithelial breast abnormality or abnormalities and would be evidence of genetic instability in *BRCA* carrier. So far, however, *BRCA1* has been shown

* Corresponding author. Tel.: (202) 444-0759; fax (202) 444-1770.
E-mail address: haddadb1@georgetown.edu (B.R. Haddad).

0165-4608/03/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/S0165-4608(03)00282-6

to be haploid sufficient only in the context of embryonic fibroblasts of mice bearing a knockout of one of its alleles [6]. An early manifestation of genomic instability in human breast cancer may promote further LOH and gene amplification, such as amplification of *ERBB2* and *MYC*, since these alterations seem to occur throughout all stages of the disease, from ductal carcinoma in situ (DCIS) onward. [AQ3]

Other studies have shown an alteration or reduced expression of the fragile histidine triad (*FHIT*) gene on 3p14.2 in a significant fraction of breast carcinomas [7], and an association between these changes and breast tumor progression, suggesting that *FHIT* may have suppressor-like properties [8,9]. In familial breast cancer kindreds, early studies [10] found a high frequency of allelic imbalance at 3p14 relative to the frequency of allelic imbalance in the same region in sporadic breast cancers. More recent studies in *BRCA2*-positive (*BRCA2*+) tumors have found frequent *FHIT* LOH and reduced expression of the FHIT protein. [11,12].

Our objective was to provide a better understanding of early genomic alterations in high-risk *BRCA1/2* carriers and to determine whether these alterations correspond to specific histopathologic changes in the breast. One approach is to determine whether a field effect of genetic changes is present in the absence of histopathologic changes (i.e., in normal and benign tissues in the same breast with a *BRCA*-associated cancer). A second approach is to study the same changes in prophylactic mastectomy specimens from *BRCA1* carriers. We used laser capture microdissection (LCM) to selectively isolate pure mammary epithelial cell populations from 29 terminal ductal lobular units (TDLUs) and from areas with benign proliferative changes (e.g., sclerosing adenosis) from *BRCA1* carriers. In these studies, we examined both specimens with tumors and specimens from prophylactic mastectomies. The samples were evaluated for LOH using polymorphic dinucleotide microsatellite markers. These included intragenic and closely linked markers for the *BRCA1* gene, for other loci on chromosome 17, for the *BRCA2* gene (closely linked markers), for other loci on chromosome 13, and for the *FHIT* gene (intragenic) on chromosome 3p14.2. [AQ4] Overall, we performed a total of 105 analyses at different loci; LOH was detected in 59 of the 105 (56%). To our knowledge, this is the first report of LOH at the *BRCA1/2* loci and the *FHIT* gene in nonmalignant mammary epithelial tissue of *BRCA1/2* mutation carriers. Our results suggest that *BRCA* LOH is an early genetic change in the mammary epithelial cells of these high-risk patients and that this change is associated with sclerosing adenosis.

2. Materials and methods

2.1. Specimen availability

Specimens were obtained from five patients of defined *BRCA* status enrolled in the Familial Cancer Registry (Lombardi Cancer Center, Georgetown University): four *BRCA1* and one *BRCA2* mutation carrier. DNA was extracted from peripheral blood and breast tissue samples. All patients had invasive ductal carcinoma. In one case (case 1), a contralateral prophylactic mastectomy specimen was also available for analysis. In another case (case 3), the patient had a lumpectomy for invasive ductal carcinoma, followed by mastectomy due to positive surgical margins. The mastectomy specimen showed foci of DCIS with no evidence of ductal carcinoma; both specimens were available for analysis.

2.2. Histopathologic analysis

The formalin-fixed, paraffin-embedded tissues from the above specimens were examined by an expert breast pathologist (BS). Normal TDLUs were defined as compact lobules with fewer than 20 lobular buds. Lobules were categorized as sclerosing adenosis when they were double or more than double the size of the normal TDLUs, and typically had increased lobules and a central area of fibrosis.

2.3. LCM

We used LCM to obtain pure cell populations of selected areas from formalin-fixed, paraffin-embedded tissue sections. Representative slides were reviewed by the pathologist (BS), and areas of normal TDLUs and sclerosing adenosis were delineated. Consecutive sections were carefully microdissected using a PixCell laser capture microscope (Arcturus Engineering, Mountain View, CA) as described previously [13]. On average, 2000 cells were isolated from each designated area (Fig. 1).

In addition to the tumors, a total of 29 areas showing normal TDLUs (10 areas) or benign proliferative changes such as sclerosing adenosis (19 areas) were evaluated for evidence of LOH. The nontumor tissues analyzed were microdissected, using LCM, from areas adjacent to the tumor (same quadrant), from other quadrants of the same breast, and in one case (a *BRCA1* mutation carrier) from the prophylactic mastectomy specimens of the contralateral normal breast.

2.4. LOH analysis

Polymorphic dinucleotide microsatellite markers, including markers for the *BRCA1* gene (intragenic markers and closely linked markers), for other loci on chromosome 17, for the *BRCA2* gene (closely linked markers), for other loci on chromosome 13, and for the *FHIT* gene on 3p14.2 (intragenic markers), were used to evaluate tissue specimens for evidence of LOH (Table 1). These markers were chosen based on their reported high heterozygosity rate [14–16]. Prior to the analysis of LOH in the tumor and adjacent areas, each microsatellite marker from our panel was first evaluated for informativeness using DNA prepared from the patient's peripheral blood. The tumor tissue and the normal and benign areas surrounding the tumor were then evaluated for LOH at the informative markers.

4C/FPO

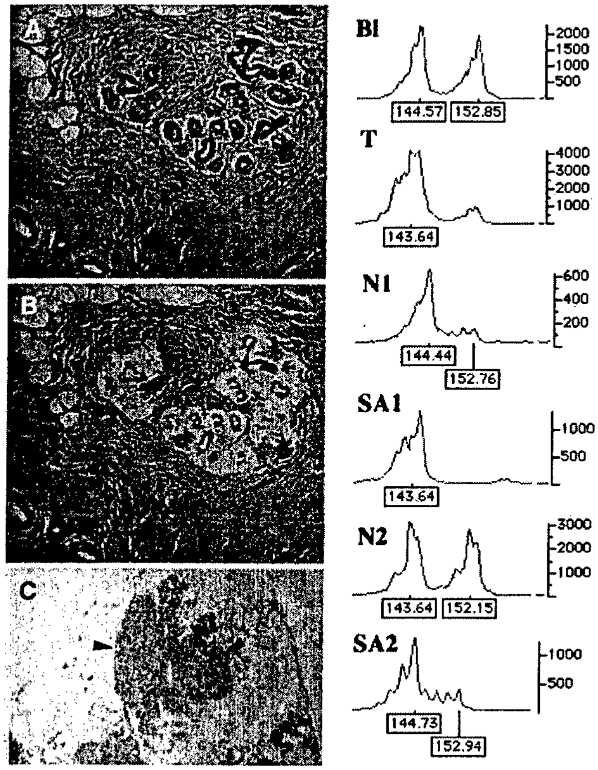
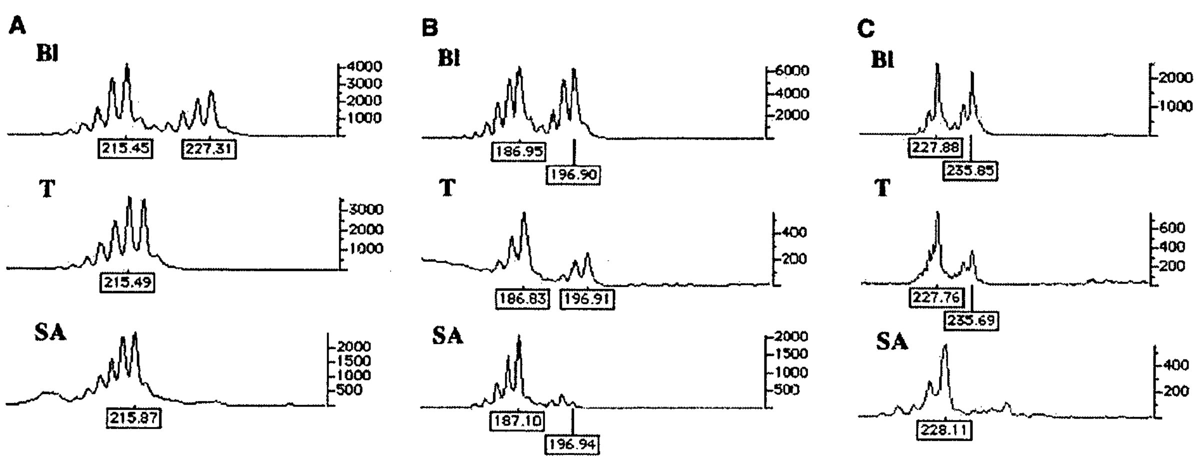


Fig. 1. (*A*) Histologically normal terminal duct lobular unit (hematoxylin–eosin stain, 20×) adjacent to the tumor, from a *BRCA1*+ patient with cancer. (*B*) Lobule after laser capture microdissection. A majority of the epithelial tissue has been microdissected. (*C*) Area with sclerosing adenosis (*arrowhead*) (hematoxylin–eosin stain, 20×), adjacent to the tumor from the same patient. The tracings on the right show the LOH analysis using the D17S855 *BRCA1* intragenic marker, performed on tissues microdissected from the same patient's specimens. Tissues studied were isolated using LCM. From top to bottom, studies in peripheral blood (Bl) show the marker to be heterozygous. LOH is detected in the tumor (T). The same allele is also lost in normal lobular tissue (N1) adjacent to the tumor and in an area with sclerosing adenosis (SA1) adjacent to the tumor. In the contralateral breast, normal tissues (N2) show no LOH, but an area with sclerosing adenosis (SA2) shows LOH for the *BRCA1* marker.

After microdissection, the cells were immediately incubated in 50 μL of digestion buffer (Arcturus) containing proteinase K (1 μg/mL) at 42°C overnight for DNA digestion. After inactivation of proteinase K (95°C for 10 minutes), 1 μL of the digestion material was directly used as a template in a 10-μL polymerase chain reaction (PCR) reaction. The PCR and cycling conditions were adapted for each primer set of our microsatellite panel. For each reaction, the genomic DNA obtained from the peripheral blood of the patient was included as normal control for LOH analysis. PCR was performed using a PTC-200 thermal cycler (MJ Research, Waltham, MA). The primer sets were obtained from Research Genetics (Huntsville, AL). The forward primers for each set were labeled using one of two fluorescent dyes, HEX or FAM.

Allele sizes were determined by electrophoresis of PCR products in 6% denaturing polyacrylamide gels and compared with ROX 500 size standards (Applied Biosystems, Foster City, CA) using an automated sequencer (ABI 377; Applied Biosystems), according to manufacturer's instructions. The fluorescent signals from the different size alleles were recorded and analyzed using Applied Biosystems Genotyper version 2.1 and GeneScan version 3.1 software. The presence of LOH was determined by at least two independent observers. For a given informative marker, LOH was defined by a decrease of either peak of at least 50%. The results were read on computer printouts [14]. Each LOH experiment was repeated at least two times, using the same DNA preparations from different PCR reactions, to evaluate the reproducibility of the results.

4C/FPO



[AQ10] Fig. 2. Other examples of LOH at the following loci markers (from left to right): D13S153, D17S785, and D3S1300 in case 5 (*BRCA2*+), case 4 (*BRCA1*+), and case 3 (*BRCA1*+), respectively. In each panel, the top tracing shows the analysis in peripheral blood (Bl), the middle tracing shows the analysis in the tumor (T), and the bottom tracing shows the analysis in an area with sclerosing adenosis.

Table 1
The panel of polymorphic dinucleotide microsatellite markers used

Marker	Site [gene]	Size, bp[a]	Heterozygozity, %
D17S786	17p12	135–157	77
TP 53	17p13	230	90
D17S849	17p13	215–253	67
D17S250	17q11.2~q12	151–169	91
D17S806	17q21	153–185	91
D17S855	17q21.2 [*BRCA1*]	145	82
D17S579	17q21.3	111–133	87
D17S785	17q24	181–207	84
D17S784	17q25	226–238	79
D13S289	13q12.1	260–276	74
D13S153	13q14.1~q14.3	212–236	82
D13S137	13q14.3	113–135	84
D13S173	13q32~q34	166–178	84
[AQ9] D3S1300	3p21.1~p14.2 [*FHIT*]	217–241	83
D3S1481	3p14.2 [*FHIT*]	104	83

[a] Expected size range of the amplified polymerase chain reaction product.

3. Results

Using the informative polymorphic dinucleotide microsatellite markers, we evaluated a total of 29 areas showing either normal TDLUs (10 areas) or benign proliferative changes such as sclerosing adenosis (19 areas) from four *BRCA1*+ patients and one *BRCA2*+ patient with breast cancer. In addition, tumor tissues were evaluated in each case. [AQ5] Overall, we performed a total of 105 analyses at different loci; of these, LOH was detected in 59 studies (56%). In the normal tissues, 15 of 30 analyses (50%) showed LOH; in the tissues with benign proliferative changes, 44 of 75 [AQ6] analyses (59%) showed LOH. Table 2 summarizes the results.

In all four cases with a *BRCA1* mutation (*BRCA1*+), we detected LOH at the *BRCA1* gene (marker D17S855) in tumor tissues and in both normal areas and areas with sclerosing adenosis. In case 1 (*BRCA1*+), a sequential loss of the *BRCA1* intragenic marker D17S855 was observed in distant areas, up to 8.7 mm from the tumor, as well as in the other three breast quadrants. We also detected LOH at this marker in the normal contralateral breast, which had been removed prophylactically (Fig. 1 and Table 3). In that same case, LOH at the D17S785 marker on the distal region of 17q was also detected in the tumor and the surrounding normal tissues. In case 2, not only was LOH at the *BRCA1* marker detected in both the tumor and the surrounding areas with sclerosing adenosis, but also at markers D17S784 and D17S785 located at the distal region of 17q. In case 3, there was LOH at the D17S855 marker in the benign areas surrounding the invasive ductal carcinoma. This same marker was also lost in normal tissues and tissues with DCIS in the same breast, which was subsequently removed by mastectomy. Markers D17S806 and D17S579, both flanking the *BRCA1* gene, were also lost, but more distant markers on the p and q arms of chromosome 17 were not lost. In Case 4, in addition to loss of the *BRCA1* gene marker, LOH was detected at the D17S785 marker located at the distal 17q region in the sclerosing adenosis areas (Fig. 2), but not in the normal area surrounding the tumor.

Table 2
Number of microsatellite marker analyses performed, with LOH detected

	Total analyses, no.	LOH found, no. (%)
Total	105	59 (56)
Normal TDLUs	30	15 (50)
Benign proliferative changes	75	44 (59)

In Case 5 (*BRCA2*+), LOH at the closely linked markers for *BRCA2* was detected in the tumor, in the surrounding areas with normal tissues, and in sclerosing adenosis (Fig. 2).

The D3S1300 marker, intragenic to the *FHIT* gene, was lost in most of the cases studied, except in case 1, where no [AQ7] LOH for the *FHIT* gene was detected. In case 3, the invasive tumor and areas surrounding it (both normal and sclerosing adenosis) showed LOH at the *FHIT* locus (Fig. 2), but no LOH was detected in the DCIS specimen, nor in the areas surrounding it.

In all cases where LOH at the *BRCA* loci was detected in the areas showing normal TDLUs or benign proliferative changes, the same allele was the one lost in the tumor, suggesting that the wild-type allele was the allele lost by LOH.

4. Discussion

In familial breast cancers associated with germline mutations in *BRCA1/2* genes, the most common mechanism of inactivation in the tumor is complete loss of the wild-type allele [2–5]. Although this is a highly reproducible observation, the correlation of such an initial LOH event with any specific histopathologic change or changes remains completely unknown. To date, no studies have systematically examined the consequences of inheritance of a mutation in the *BRCA1* or *BRCA2* genes for corresponding early changes in breast histopathology, nor have studies addressed the correlation of such early abnormalities in the breasts of *BRCA* mutation carriers with genomic gains or losses, LOH, or replication error repair instability.

In this study, we have detected LOH at multiple loci, not only in malignant regions in the breasts of *BRCA1* and *BRCA2* mutation carriers with breast cancer, but also in morphologically normal TDLUs and in tissues with benign proliferative changes (e.g., sclerosing adenosis) from the same patients. Most of the losses detected in normal and benign tissues were also present in the tumor tissues, suggesting that these nonmalignant tissues already harbor significant genetic alterations that may predispose to malignant transformation. Our results support the hypothesis that there is a field effect of genomic aberrations, in which some of the genetic changes detected in *BRCA*-associated cancers are also present in the nonmalignant areas adjacent to the tumors, as well as in the *BRCA*+ contralateral prophylactic

Table 3
Summary of LOH analyses at the *BRCA1* marker (D17S855) in tissues isolated from case 1 (*BRCA1*+) from different areas of the breast with tumor and of the contralateral breast removed prophylactically

	Quadrant	Tissue	Distance from Tumor	LOH at D17S855
Breast with tumor	Upper outer quadrant	Tumor		LOH
Same	Same	Sclerosing adenosis	0.1 mm	LOH
Same	Same	Sclerosing adenosis	0.3 mm	LOH
Same	Same	Normal	0.8 mm	LOH
Same	Same	Normal	3.5 mm	No LOH
Same	Same	Sclerosing adenosis	6 mm	LOH
Same	Same	Normal	8.7 mm	LOH
Same	Upper inner quadrant	Sclerosing adenosis		LOH
Same	Lower outer quadrant	Sclerosing adenosis		LOH
Same	Lower inner quadrant	Sclerosing adenosis		LOH
Contralateral breast, no tumor		Normal		No LOH
Same		Sclerosing adenosis		LOH

mastectomy specimens. The concept of field cancerization was originally described by Slaughter et al. in 1953 [17] in their study of oral cancer and was used in reference to the presence of preneoplastic histologic changes at multiple sites. More recent studies have described the molecular changes associated with the multistep development and progression of cancer [18]. In several other studies, morphologically normal tissues adjacent to tumors have been shown to harbor molecular changes such as LOH, microsatellite alterations [19,20], chromosomal instability [21], and mutations in the *TP53* gene [22]. Such studies elegantly provide a molecular definition of the old concept of field cancerization, and may indicate the presence of a field of genetically altered cells that may be at higher risk of cancer transformation.

Additional reports, including our own, support this concept of a field effect of genetic alterations in mammary tissue specimens from *BRCA1/2* mutation carriers. Previous studies of both familial and sporadic breast tumors have shown that genetic changes can be detected in morphologically normal appearing breast tissues. LOH has been detected in morphologically normal lobules, adjacent to sporadic breast cancer, suggesting the presence of a field effect of preexisting genomic damage in the gland, giving rise to the tumor [19]. LOH was also detected in hyperplasias (usual ductal hyperplasia and atypical ductal hyperplasia) from both cancerous and noncancerous breasts [23–25]. In some cases, the allelic losses observed in these hyperplasias were similar to the ones frequently seen in the breast carcinomas, suggesting a preneoplastic condition, a genetically altered precursor cell shared with an adjacent carcinoma, or both. More recently, normal breast tissues adjacent to invasive ductal carcinomas from 12 breast cancer patients (6 monozygotic twin pairs) who were negative for mutations in the *BRCA1/2* genes were analyzed for LOH on chromosomes 1, 13, and 17. Seventeen LOHs were observed in the normal tissues, 14 of which were present in the corresponding breast tumor [26]. In addition, two other studies have shown cytogenetic abnormalities in prophylactic mastectomy specimens characterized by hyperplasia, without atypia, from patients with a positive family history of breast cancer (but of unknown *BRCA* status) [27,28].

We have also examined the correlation of *BRCA1* LOH with *FHIT* LOH. Deletions of 3p14 have been observed in benign proliferative breast disease [27,29], and one report has shown that the *FHIT* gene was homozygously deleted in 2 cases of benign proliferative breast disease associated with 3p14 cytogenetic rearrangements and familial breast cancer [30], suggesting that loss of the *FHIT* gene may be an early event in mammary carcinogenesis. Specifically, we examined whether *FHIT* LOH occurs independent of *BRCA1* LOH and, if so, what are the histopathologic associations with such an observation. Although LOH at *BRCA1* and *FHIT* loci commonly occurred in the same specimens, we have occasionally observed *BRCA1* LOH in the absence of *FHIT* LOH—but never the reverse. This observation is suggestive of haploid sufficiency of *BRCA1* gene for protection of breast epithelial cells from chromosomal instability. Alternatively, if *FHIT* LOH were common in the absence of *BRCA1* LOH, we might postulate that *FHIT* LOH is an initial or early genetic event to which *BRCA1* carriers are predisposed. In such a scenario, *FHIT* LOH could even predispose the genome to other genetic changes, including *BRCA1* LOH. Our study does not support this model.

To our knowledge, this is the first report of genetic changes (LOH) in normal TDLUs and in benign tissues from breast cancer patients who are carriers of mutations in the *BRCA1* and *BRCA2* genes. Such changes may represent the earliest detectable genomic aberrations that occur during the development and progression of breast cancer in these high-risk patients. We conclude that LOH at the relevant *BRCA* loci is an early event in *BRCA* mutation carriers, and may be detected in nonmalignant cells. Although the significance of this finding needs further evaluation in larger studies, ultimately it (i.e., LOH at *BRCA* loci in nonmalignant cells of *BRCA*+ patients) might be used as a marker [AQ8] for elevated risk of malignant transformation in these high-risk patients. The identification of such early genetic changes

will improve our understanding of the mechanisms of tumorigenesis, and may be useful for developing molecular markers for early detection and diagnosis of hereditary breast cancer in *BRCA1/2* carriers.

Acknowledgments

This work was supported by a U.S. Department of Defense (DOD) Breast Cancer Research Grant (DAMD17-99-1-9193) to BRH. We thank the Familial Cancer Registry and the Histopathology and Tissue Shared Resources of Lombardi Cancer Center, which are partially supported by National Institutes of Health Grant 1P30-CA-51008 (Cancer Center Support Grant, to Lombardi Cancer Center). We also thank Drs. Deborah Boles and Janice Rone for critical reading of the manuscript.

References

[1] Ingvarsson S. Molecular genetics of breast cancer progression. Semin Cancer Biol 1999;9:277–88.
[2] Cornelis RS, Neuhausen SL, Johansson O, Arason A, Kelsell D, Ponder BA, Tonin P, Hamann U, Lindblom A, Lalle P. The Breast Cancer Linkage Consortium. High allele loss rates at 17q12-q21 in breast and ovarian tumors from BRCA1-linked families. Genes Chromosomes Cancer 1995;13:203–10.
[3] Staff S, Nupponen NN, Borg A, Isola JJ, Tanner MM. Multiple copies of mutant *BRCA1* and *BRCA2* alleles in breast tumors from germline mutation carriers. Genes Chromosomes Cancer 2000;28:432–42.
[4] Nathanson KN, Wooster R, Weber BL. Breast cancer genetics: what we know and what we need. Nat Med 2001;7:552–6.
[5] Osorio A, de la Hoya M, Rodriguez-Lopez R, Martinez-Ramirez A, Cazorla A, Granizo JJ, Esteller M, Rivas C, Caldes T, Benitez J. Loss of heterozygosity analysis at the *BRCA* loci in tumor samples from patients with familial breast cancer. Int J Cancer 2002;99:305–9.
[6] Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, Hennighausen L, Wynshaw-Boris A, Deng CX. Conditional mutation of *BRCA1* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat Genet 1999;22:37–43.
[7] Negrini M, Monaco C, Vorechovsky I, Ohta M, Druck T, Baffa R, Huebner K, Croce CM. The *FHIT* gene at 3p14.2 is abnormal in breast carcinomas. Cancer Res 1996;56:3173–9.
[8] Campiglio M, Pekarsky Y, Menard S, Tagliabue E, Pilotti S, Croce CM. FHIT loss of function in human primary breast cancer correlates with advanced stage of the disease. Cancer Res 1999;59:3866–9.
[9] Gatalica Z, Lele SM, Rampy BA, Norris BA. The expression of Fhit protein is related inversely to disease progression in patients with breast carcinoma. Cancer 2000;88:1378–83.
[10] Bergthorsson JT, Eiriksdottir G, Barkardottir RB, Egilsson V, Arason A, Ingvarsson S. Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3. Hum Genet 1995;96:437–43.
[11] Ingvarsson S, Geirsdottir EK, Johannesdottir G, Sigbjornsdottir BI, Eiriksdottir G, Ragnarsson G, Agnarsson BA, Gudmundsson J, Jonasson JG, Sigurdsson A, Egilsson V, Barkardottir RB. High incidence of loss of heterozygosity in breast tumors from carriers of the BRCA2 999del5 mutation. Cancer Res 1998;58:4421–5.
[12] Ingvarsson S, Agnarsson BA, Sigbjornsdottir BI, Kononen J, Kallioniemi OP, Barkardottir RB, Kovatich AJ, Schwarting R, Hauck WW, Huebner K, McCue PA. Reduced Fhit expression in sporadic and *BRCA2*-linked breast carcinomas. Cancer Res 1999;59:2682–9.
[13] Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA. Laser capture microdissection. Science 1996;274:998–1001.
[14] Kerangueven F, Noguchi T, Coulier F, Allione F, Wargniez V, Simony-Lafontaine J, Longy M, Jacquemier J, Sobol H, Eisinger F, Birnbaum D. Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. Cancer Res 1997;57:5469–74.
[15] Phelan CM, Borg A, Cuny M, Crichton DN, Baldersson T, Andersen TI, Caligo MA, Lidereau R, Lindblom A, Seitz S, Kelsell D, Hamann U, Rio P, Thorlacius S, Papp J, Olah E, Ponder B, Bignon YJ, Scherneck S, Barkardottir R, Borresen-Dale AL, Eyfjord J, Theillet C, Thompson AM, Devilee P, Larsson C. Consortium study on 1280 breast carcinomas: allelic loss on chromosome 17 targets subregions associated with family history and clinical parameters. Cancer Res 1998;58:1004–12.
[16] Euhus DM, Maitra A, Wistuba II, Alberts A, Albores-Saavedra J, Gazdar AF. Loss of heterozygosity at 3p in benign lesions preceding invasive breast cancer. J Surg Res 1999;83:13–8.
[17] Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium. Cancer 1953;6:963–8.
[18] Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67.
[19] Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science 1996;274:2057–2059.
[20] Tabor MP, Brakenhoff RH, van Houten VM, Kummer JA, Snel MH, Snijders PJ, Snow GB, Leemans CR, Braakhuis BJ. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. Clin Cancer Res 2001;7:1523–32.
[21] Hittelman WN. Genetic instability in epithelial tissues at risk for cancer. Ann N Y Acad Sci 2001;952:1–12.
[22] Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidransky D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med 1995;332:429–35.
[23] O'Connell P, Pekkel V, Fuqua S, Osborne CK, Allred DC. Molecular genetic studies of early breast cancer evolution. Breast Cancer Res Treat 1994;32:5–12.
[24] O'Connell P, Pekkel V, Fuqua SA, Osborne CK, Clark GM, Allred DC. Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. J Natl Cancer Inst 1998;90:697–703.
[25] Washington C, Dalbegue F, Abreo F, Taubenberger JK, Lichy JH. Loss of heterozygosity in fibrocystic change of the breast: genetic relationship between benign proliferative lesions and associated carcinomas. Am J Pathol 2000;157:323–9.
[26] Forsti A, Louhelainen J, Soderberg M, Wijkstrom H, Hemminki K. Loss of heterozygosity in tumour-adjacent normal tissue of breast and bladder cancer. Eur J Cancer 2001;37:1372–80.
[27] Petersson C, Pandis N, Mertens F, Adeyinka A, Ingvar C, Ringberg A, Idvall I, Bondeson L, Borg A, Olsson H, Kristoffersson U, Mitelman F. Chromosome aberrations in prophylactic mastectomies from women belonging to breast cancer families. Genes Chromosomes Cancer 1996;16:185–8.
[28] Teixeira MR, Pandis N, Gerdes AM, Dietrich CU, Bardi G, Andersen JA, Graversen HP, Mitelman F, Heim S. Cytogenetic abnormalities in an in situ ductal carcinoma and five prophylactically removed breasts from members of a family with hereditary breast cancer. Breast Cancer Res Treat 1996;38:177–82.
[29] Dietrich CU, Pandis N, Teixeira MR, Bardi G, Gerdes AM, Andersen JA, Heim S. Chromosome abnormalities in benign hyperproliferative disorders of epithelial and stromal breast tissue. Int J Cancer 1995;60:49–53.
[30] Panagopoulos I, Pandis N, Thelin S, Petersson C, Mertens F, Borg A, Kristoffersson U, Mitelman F, Aman P. The *FHIT* and *PTPRG* genes are deleted in benign proliferative breast disease associated with familial breast cancer and cytogenetic rearrangements of chromosome band 3p14. Cancer Res 1996;56:4871–5.